IJBST

ISSN: 0974 – 3987
IJBST (2010), 3(6):61-67

Optimization of Enzymatic Hydrolysis of Rice Starch by Immobilized α-Amylase using Response Surface Methodology

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ABSTRACT:
Enzymatic hydrolysis of starch from natural sources finds potential application in commercial production of alcoholic beverage and bioethanol. This work deals with the modeling of the enzymatic hydrolysis of rice waste using immobilized α-amylase. Optimization strategies (starch concentration, enzyme concentration, temperature and time) were evaluated by the use of Response Surface Methodology (RSM). The experimental result on enzymatic hydrolysis of rice waste was subjected to multiple linear regression analysis using MINITAB 14 software. The most significant effect of starch concentration, temperature and time were found on hydrolysis of rice starch by immobilized α-amylase enzyme. The statistical significance of the model was validated by F-test for analysis of variance ($p \leq 0.01$).The maximum glucose produced 1.16 mg/ml at starch concentration 8.17(%), enzyme concentration 1.34 (%), temperature 34°C, time 96.41 min.

Key words: Enzymatic hydrolysis, response surface methodology, regression analysis, immobilization.

INTRODUCTION

Starch is a major storage product of many economically important crops such as wheat, rice, maize, tapioca, and potato. In the past few decades, research has been focused on the use of starch-converting enzymes in the production of hexoses (maltodextrin, glucose and fructose syrups) [1, 2]. So far, four groups of starch converting enzymes have been identified namely, endoamylases, exoamylases, debranching enzymes and transferases.

Endoamylases cleave α -1, 4 glycosidic bonds in a random fashion present in the amylose or amylopectin chain (component sugars of starch) and α -amylase is a well-known endoamylase [3-7]. Stabilization of the enzyme would reduce the need to purchase new enzymes and hence reduce the cost of bioprocesses. One of the most effective ways to achieve stabilization is by the use of immobilized enzyme [8].

Multipoint covalent attachment to solid supports has been used to stabilize several industrial enzymes [9]. The formation of the rigid enzyme–support linkage provides both kinetic and thermodynamic stabilization of the three-dimensional structure of the active catalytic site. The immobilized enzyme molecules may also be stabilized against denaturing agents that promote unfolding processes that can destroy the active site [10]. Immobilization by entrapment is frequently used to provide high enzyme efficiencies, but entrapment is only suitable for soluble substrates [11].

RSM is a collection of mathematical and statistical techniques widely used to determine the effects of several variables and to optimize different biotechnological processes [12-16]. Empirical models and statistical analysis are extremely important to elucidate basic mechanisms in complex situations, thus providing better process control and understanding. In most RSM problems, the relationship between the response and independent variables is unknown. Thus the first step in RSM is to approximate the process to a function (f), in some region of the independent variables. If the response is well modeled by a linear function of the independent variables, then the approximating function is a first-order model. If there is curvature in the system or in the optimum region, then a polynomial of higher degree, such as a second-order model, must be used to approximate the response. The main objective of RSM is to determine the optimum operational conditions for the system or to determine a region that satisfies the operating specifications. Optimization of enzymatic hydrolysis of rice starch by the classical method involves changing one independent variable (starch concentration, enzyme concentration ,temperature , time) while maintaining all others at a fixed level which is extremely time consuming and expensive for a large number of variables. To overcome this difficulty, experimental factorial design and response methodology can be employed to optimize the enzymatic hydrolysis of rice starch.

MATERIAL AND METHODS

Materials

The boiled rice water was used as a carbon source. It was obtained by cooking rice at 80°C with extra water. Then scoop out the boiled water. The scoop out rice waste water solution used through out the study was prepared by dissolving required quantity (based on table 2) in distilled water. The fungal 1,4-Alpha-D-glucan- glucanohydrolase (α-amylase CAS NO. 9001-19-8, 1:2000 IP Units) produced from *Aspergillus oryzae* source used in the present study was obtained from HiMedia Laboratories Pvt. Ltd, Mumbai, India.

Immobilization of enzyme

The alginate entrapment of enzyme was performed according method of Johnson and Flink [17]. Sodium alginate solution (3%) (Loba Chemie, Mumbai, India) was prepared by dissolving sodium alginate in boiling water and mixed with enzyme (based on table2). Both alginate slurry and enzyme suspension were mixed and stirred for 10 minutes to get a uniform mixture. The slurry was taken into a syringe and added drop wise into 0.2 M $CaCl_2$ solution and kept for curing at 4°C for 1h. The cured beads were washed with sterile distilled water 3 to 4 times then used for hydrolysis of starch.

OPTIMIZATION BY RESPONSE SURFACE METHODOLOGY

RSM explores the relationships between several explanatory variables and one or more response variables. The main aim for RSM is to use a sequence of designed experiments to obtain an optimal response. The statistical method was obtained using Central Composite Design (CCD) with four independent variables (starch concentration, enzyme concentration, temperature, time). Each variable in this design was studied at three different levels (Table 1). All variables were taken at a central coded value considered as zero. The minimum and maximum ranges of variables were used and the full experimental plan with respect to their values in coded from is listed in Table 2. All experiments on completion, the average of glucose production was taken as the dependent variable or response. A full polynomial model obtained by a multiple regression technique for four variable using MINITAB 14 to determine the optimal response region of the glucose production from hydrolysis of starch by α-amylase enzyme.

ESTIMATION OF GLUCOSE

Experiments were conducted according to central composite design (Table 2) to study the interaction of starch concentration, temperature, enzyme concentration and time on enzymatic hydrolysis of starch by immobilized α-amylase enzyme. All the experiments were conducted in 250 ml Erlenmeyer Flasks constantly mixed by magnetic stirrer. The glucose concentration (Y) from hydrolysis of starch was measured as reducing sugar concentration using the DNS (3, 5-dinitrosalicylic acid) method. This method is adequate because glucose is the unique reducing sugar of the starch [18].

RESULTS AND DISCUSSION

Hydrolysis of starch by α-amylase from *Aspergillus oryzae*

The statistical method was obtained using CCD with four independent variables (starch concentration, enzyme concentration, temperature, time). These values which resulted in the production of 1.16 mg/ml of glucose were fitted in the model CCD falling under RSM. Glucose production (i.e. the response) of the experiment (CCD) for each individual run along with the predicted responses is presented in Table 2. The maximum glucose production, 1.16 mg/ml was achieved in starch concentration 8.17(%), enzyme concentration 1.34 (%), temperature 34°C, time 96.41 min.

The result obtained after CCD were then analyzed by standard analysis of variance (ANOVA), which gave the following regression equation (in terms of coded factors) –

$$Y=-4.416+0.356X_1+0.594X_2+0.106X_3+0.031X_4-0.018X_1^2-0.457X_2^2-0.000X3^2-0.000X_4^2-0.013X_1X_2-0.002X_1X_3+0.000X_1X_4-0.022X_2X_3-0.000X_2X_4-0.000X_3X_4..(1)$$

where Y: glucose concentration, X_1: starch concentration, X_2: enzyme concentration, X_3: temperature, X_4: time.

The student's t-test was used to determine the significance of the regression coefficients. The results of statistical analysis including the regression coefficient, t and p values for linear, quadratic and combined effects of the variables were given in the table 3. The p-values are used as a tool to check the significance of each of the coefficients and to understand the interactions between the best variables. The most significant variable for (X_1) starch concentration, (X_3) temperature and (X_4) time and square variable of (X_1 X_1) starch concentration (X_2 X_2) enzyme concentration, (X_4 X_4) time and interaction of (X_3 X_4) temperature and time terms.

The ANOVA (Table 4) gives the linear and square terms in second order polynomial Model (equation 1) were highly significant ($p<0.01$) and adequate to represent the relationship between glucose production (mg/ml) and starch concentration, enzyme concentration, temperature and time. The R^2 value 0.932 for glucose production, point to the accuracy of the model. The R^2 value provides a measure of how much variability in the observed response values can be explained by the experimental factors and their interactions. The R^2 value is always between 0 and 1. The closer the R^2 value is to 1.00, the stronger the model is and the better it predicts the response. The model *F*-value of 2475.40 for glucose production implied that the model is significant. Values of 'Pred > F' less than 0.05 indicated that the model terms are significant. The 'Lack of fit *F*-value' 0.001 for glucose production implied the lack of fit is insignificant and the model is adequate.

IJBST

ISSN: 0974 – 3987
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Table 1. Experimental range and levels of the independent variables

Variables	-1	0	1
Starch con (w/v)%, X_1	3	6	9
Enzyme con (w/v)%, X_2	0.5	1	1.5
Temperature (°C), X_3	30	40	50
Time (min), X_4	40	80	120

Table 2. Central composite design consisting of 31 experiments for the study of four experimental Factors in coded units along with experimental and predicted glucose concentration

Std Order	X1	X2	X3	X4	Glucose concentration(mg/ml)	
					Experimental	Predicted
1	-1	-1	-1	-1	0.028	-0.187
2	1	-1	-1	-1	0.169	0.150
3	-1	1	-1	-1	0.209	0.124
4	1	1	-1	-1	0.478	0.381
5	-1	-1	1	-1	0.232	0.189
6	1	-1	1	-1	0.100	0.226
7	-1	1	1	-1	0.941	0.955
8	1	1	1	-1	0.914	0.913
9	-1	-1	-1	1	0.472	0.517
10	1	-1	-1	1	0.042	0.939
11	-1	1	-1	1	1.022	0.807
12	1	1	-1	1	1.063	1.150
13	-1	-1	1	1	0.277	0.286
14	1	-1	1	1	0.280	0.408
15	-1	1	1	1	0.969	1.013
16	1	1	1	1	0.947	1.074
17	0	0	0	0	0.000	0.191
18	0	0	0	0	0.718	0.571
19	0	-2	0	0	0.100	0.113
20	0	2	0	0	1.059	1.090
21	0	0	-2	0	0.248	0.527
22	0	0	2	0	1.061	0.287
23	0	0	0	-2	0.011	0.148
24	0	0	0	2	1.061	1.013
25	0	0	0	0	1.058	1.060
26	0	0	0	0	1.064	1.060
27	0	0	0	0	1.062	1.060
28	0	0	0	0	1.057	1.060
29	0	0	0	0	1.059	1.060
30	0	0	0	0	1.053	1.060
31	0	0	0	0	1.064	1.060

Table 3. Estimated regression coefficients of second order polynomial model for Optimization of glucose production

Coefficient	Estimated Coefficient	t- value	p-value
β_0	-4.416	-5.381	0.000
β_1	0.356	4.652	0.000*
β_2	0.594	1.291	0.215
β_3	0.106	3.859	0.001*
β_4	0.031	5.519	0.000*
β_{11}	-0.018	-5.711	0.000*
β_{22}	-0.457	-3.851	0.001*
β_{33}	-0.000	-3.223	0.005*
β_{44}	-0.000	-4.031	0.001*
β_{12}	-0.013	-0.506	0.620
β_{13}	-0.002	-1.888	0.077
β_{14}	0.000	0.537	0.599
β_{23}	0.022	2.868	0.011
β_{24}	-0.000	-0.131	0.898
β_{34}	-0.000	-3.825	0.001*

IJBST

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Table 4. Analysis of variance (ANOVA) of second order polynomial model for optimization of glucose production by the hydrolysis of starch (R^2=93.2%)

Factors	Degrees of Freedom	Sum of Squares	Mean Square	F-value	P-value
Regression	14	5.032	0.359	14.26	<0.001
Linear	4	2.905	0.307	12.21	<0.001
Square	4	1.446	0.361	14.35	<0.001
Interaction	6	0.679	0.113	4.50	0.007
Residual Error	16	0.403	0.025		
Lack-of-Fit	10	0.403	0.040	2475.40	<0.001
Pure Error	6	0.000	0.000		
Total	30	5.435			

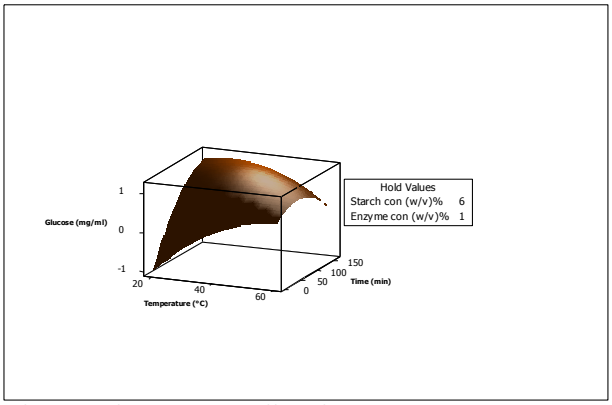

Fig.1 Response Surface plot of the combined effect of temperature and time on glucose concentration.

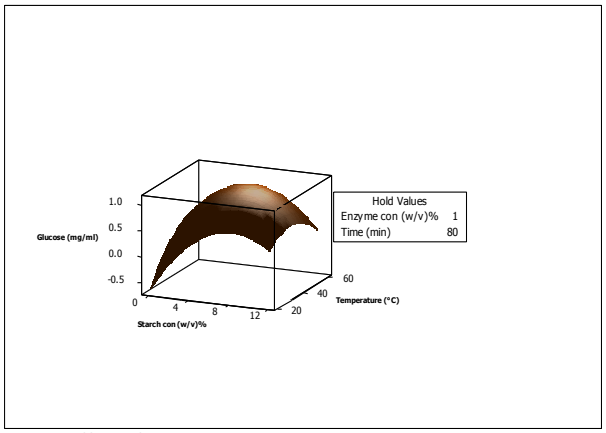

Fig.2 Response Surface plot of the combined effect of temperature and starch concentration on glucose concentration

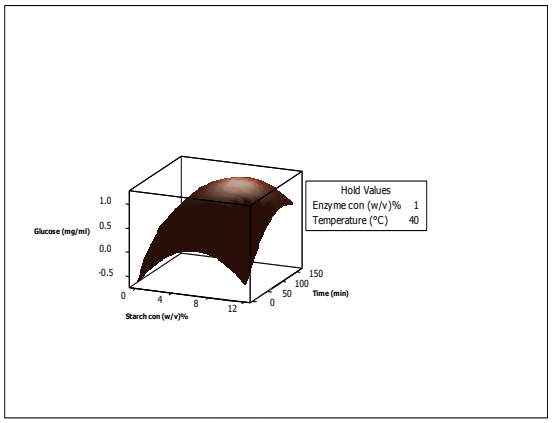

Fig.3 Response Surface plot of the combined effect of time and starch concentration on glucose concentration

IJBST

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IJBST (2010), 3(6):61-67

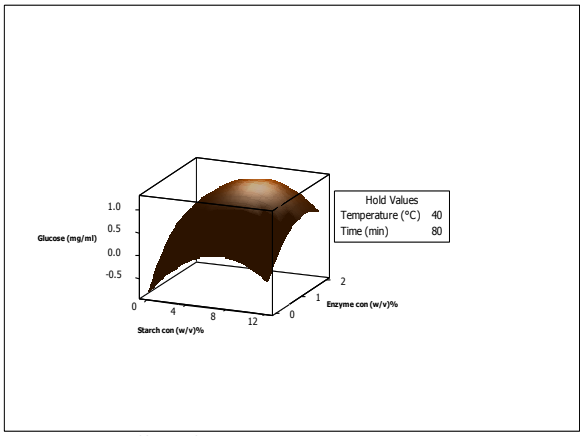

Fig.4 Response Surface plot of the combined effect of starch concentration and enzyme concentration on glucose concentration

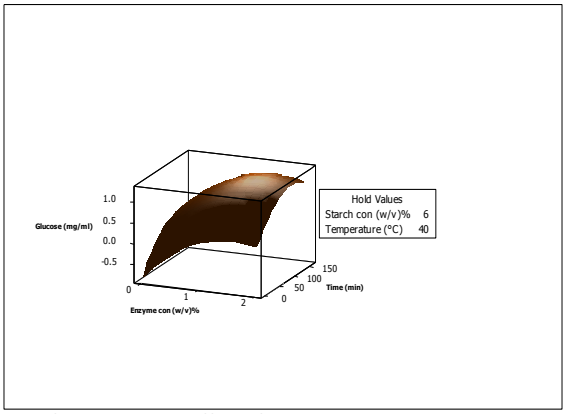

Fig.5 Response Surface plot of the combined effect of time and enzyme concentration on glucose concentration

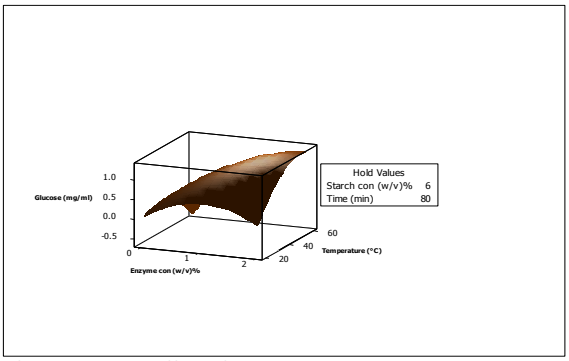

Fig.6 Response Surface plot of the combined effect of temperature and enzyme concentration on glucose concentration

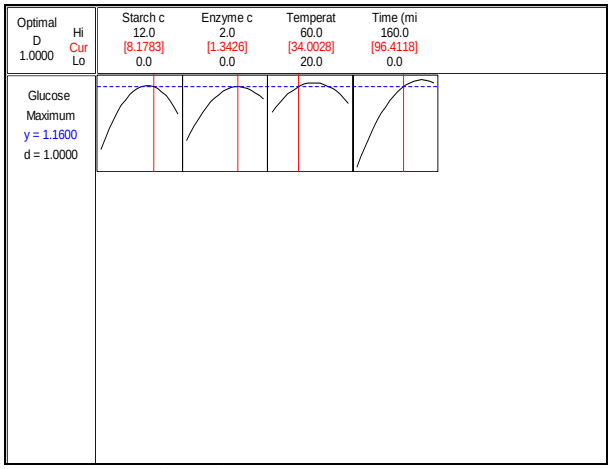

Fig.7 Optimization plot surface plot of the combined effect of temperature and time on glucose concentration

The response surface plots describing combined effect between pair of variables on hydrolysis of starch were given in figure 1 to 6. Fig1. The surface plot explain that at glucose concentration maximum at temperature towards increase to middle value to high value and middle value of time, while starch concentration and enzyme concentration are constant.Fig.2.shows the effect of starch concentration and temperature on glucose concentration while other variable (enzyme concentration and time) was fixed at middle, glucose concentration high at middle level of starch concentration and temperature where it was decrease at low and high level of starch concentration and temperature. Fig.3 shows that high and low level of starch concentration, time has no significant on glucose production and increase production was absorbed at middle level of starch concentration and time. Fig 4.shows the interaction effect of starch and enzyme concentration on glucose concentration while other factor are constant (temperature and time) while glucose production maximum at middle level of starch and enzyme concentration where at minimum at low and high level of starch, enzyme concentration. The glucose production significant level at middle value of enzyme concentration and time and low and high value insignificant in Fig 5.

From Fig 6.observed that middle level enzyme concentration and higher level temperature maximum glucose obtained where at lower level of temperature and high, low level enzyme concentration low amount of glucose produced remaining variable are constant.

CONCLUSION

Commercial fungal α-amylase was immobilized by entrapment method for hydrolysis of rice starch waste. Statistical optimization of enzymatic hydrolysis for glucose production from food waste has been successfully carried out using RSM based on the 2^4 factorial CCD. The optimal conditions for reducing sugar production were determined as follows starch concentration 8.17(%), enzyme concentration1.34(%), temperature34°C, time 96.41 min Under these conditions, experimental results of reducing sugar concentration were obtained as 1.16(mg/ml) (Fig.7). This design proved to be useful in the optimization of enzymatic hydrolysis for glucose production from food waste.

ACKNOWLEDGEMENT

Authors wish to acknowledge the authorities of Madha Engineering College, Chennai – 600069, for their support to carry out this research work.

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